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IMMUNOGENIC ROLE OF PROPOLIS INCORPORATED LIPOPOLYSACCHARIDE ANTIGEN OF *SALMONELLA ENTERICA* SEROVAR *TYPHIMURIUM* ISOLATED FROM HUMAN INFECTION

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ABSTRACT : In this study, total (75) of clinical specimens were collected obtained frompatients suffer from severe diarrhea that was associated with fever as diagnostic by physician,these specimen take from different location of Hilla city during a period from (August 2017 to November 2017).Results showed out of 75 studied specimens of patients, 14 specimens were *Salmonella* positive (18.66%). Fourteen *Salmonella* isolate were isolated and identified using bacterial culturing on selective media and subjected for further confirming tests such as API-20E, *16S rRNA* gene amplification, all method resultreferred to all isolates belong to *Salmonella* sp. One isolate were sent to registration of their sequences in Gene Bank-NCBI for diagnostic of this isolate on species level and obtaining accession numbers (MH109313.1) for *Salmonella enterica* serovar Typhimurium. Therefore, we focused on this bacteria to assess the role of *S. enterica* serovar Typhimurium lipopolysaccharide's (LPS) as vaccine against salmonella infections. SoLPS from *S. enterica* serovar Typhimurium, was extracted by proteinase K-Hot –phenol method. Separation of LPS over SDS-PAGE gel followed by silver staining was used to detect and visually characterize the purified LPS, LPS were conjugated with propolis to determine the role of PLPS-conjugate as a potential vaccine against *S. enterica* serovar Typhimurium in albino mice, and evaluate immune responses. Dot blot method was used to detected the antibody titer for the sera of vaccinated groups The results was appeared the most sample of vaccinated animals gives significant result as high titer antibody and we found that serum (polyclonal antibody) of mice immunized by LPS and PLPS able to recognize each (Ag-Ab complex) by using dot blot technique.

Key words : Propolis, lipopolysaccharide, *Salmonella enterica*, Typhimurium and human.

INTRODUCTION

Salmonellosis is disease caused by group of bacteria belong to genus salmonella that can infect human and animals. So consider, one of the most, common, foodborne diseases, worldwide accounting, around 93.8 million foodborne. Illnesses and 155,000 deaths per year worldwide Salmonella infection consider a serious problem to public health and being throughout the world so lead to substantial economic loss result from mortality, morbidity and poor growth hazard of transmitting food poisoning with gastroenteritis, to human. People at risk for serious complications due to salmonella food poisoning, include older adults pregnant women, infants children and people who have compromised immune system (Nesa *et al*, 2011).

Salmonella spp. appear to cause a higher morbidity and mortality. rate among infected animals than other non-typhoid Salmonella infection (Valenzuela *et al*, 2017). *Salmonella typhimurium* is the most important frequently isolated from global food – borne outbreaks, poultry are one of the most important reservoirs of salmonella that can be transmitted to human, through the food chain, food safety and antibiotic resistance, are the aspect, of the threat *S. typhimurium* recent reports of snowballing incidence of multiple antibiotics resistant strain of *S. typhimurium.* in human and animals have posed a major emerging public health issue, of international concern and this dramatic, increasing in drug resistance will complicate the options available, in the treatment of salmonellosis (Mohammed, 2017).

Vaccination is a prefer and convenient method for Salmonella prevention and control. Also vaccination has been implemented in several countries. There are a number of live and killed *Salmonella* vaccines used commercially worldwide. Live, attenuated vaccines are generally accepted to be more worthwhile in controlling salmonellosis than killed bacterins. Its generally accepted

that two types of immune response are involved in defense against salmonellosis cellular and humoral. Since in attempts to control salmonellosis by vaccination living vaccines have been superior to nonviable ones cellular immunity has been considered to be of greater, importance for resistance it has been firmly established in mice. (Chansiripornchai, 2017).

Bacteriam or fractions therefor can give substantial levels of protection when used as vaccines the specificity of these vaccines hasbeen attributed tothe O antigen of the vaccine strain (Coward *et al*, 2014). Several studies have demonstrated that the lipopolysaccharide (LPS) of the *S. typhimurium* is a key component associated with Bacterial virulence butin terms of vaccine potential the LPS itself isn't very immunogenic and as a result attempts have been ended at synthesizing a vaccine, which incorporates *Salmonella* LPS antigenic determinants but is devoid of the toxic properties inherent in the lipid A moiety of the LPS through the covalent attachment to carrier molecules there were many carrier molecules used as adjuvant such as liposomes (Christensen *et al*, 2011). Outer membrane vesicles (OMV) (Mitra *et al*, 2016). Recently, developed generalized modules for membrane antigens (GMMA) that are genetically derived blebs seem attractive perspective as vaccine candidates, especially in the developing countries (Rossi *et al*, 2016).

In this study, we attempt to used the natural constitutes as adjuvant such as propolis. Propolis have received a considerable amount of attention as carriers for the delivery of a wide variety of biologically active substances to cells and tissues *in vitro* and *in vivo*, but there is no study used as immunological adjuvants for the enhancement or modulation of immuneresponses.

MATERIALS AND METHODS

Specimens collection

The study was conducted in Babylon province, a total of 75 Stool specimens were collected from 75 patients suffer from fever and diarrhea after diagnostic by physician. The stool specimens were received in sterile plastic containers where a loop full from each specimens was inoculated into a sterile tube containing Brian Heart infusion broth and transferred to microbiology lab. in the Department of Microbiology, Veterinary Medicine, AL-Qasim Green University, Qadisiyah to investigated and demonstrating the occurrence of *Salmonella* spp in human stool.

Isolation and identification

Isolation

Specimens were collected and transported to the laboratory in Brian Heart infusion broth to allow the multiplication of bacteria. Theywereincubated for 24–48 hours at 37°C. The broth culture was aseptically streaked on Salmonella-Shigella Agar (SSA), plates were incubated at 37°C for 18–48 hours, after which, they were examined for colonies typical of Salmonella. Suspect colonies were streaked on nutrient agar plates to obtain pure cultures, which were subjected to another media for confirming the identification (Cheesbrough, 2002).

Identification

Identification of *Salmonella* species was done microscopically by using Gram's stain where smear was taken from bacteria and applied on it the steps of gram stain and examineted under microscope where was gram positive or negative (Collee *et al*, 1996).

Suspect Salmonella colonies that streaked on Salmonella Shigella Agar (SSA) plates, Bismuth Sulphite Agar (BSA), MacCkonkey agar and Chromogenic agar plates incubated at 37°C for 18–48 hr. for study the phenotypic characters of suspected salmonella isolates, after which, they were examined for colonies, typical, of *Salmonella* were subjected, to confirming test for confirm the identification. All suspected isolates were confirming by using, the analytical, profile, index (A-P-I) 20E kit, molecular identification vai *16SrRNA* amplification by following the instructions of the manufacturer (Nesa *et al*, 2011) and to diagnosis on species level the obtained nucleotide sequence of 16S *rRNA* gene for one isolates numerical salmonella 1(S1) was processed through Finch TV software. Analysis of the PCR product sequence was analyzed using BLAST N (Basic Local Alignment Search Tool, http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi) that is an online programme belonging to NCBI (National Center for Biotechnology Information) to determine the hits of subjects sequences deposited in the international nucleotide databases (e.g., GenBank, EMBL, DDJD, etc.) giving the best matching with the query sequence.

Extraction and purification of lipopolysaccharide (LPS)

Bacterial cells were pull out with hot aqueous phenol method as described by Westphal and Jann (1965), with a minor modification in order to ease pure LPS extraction (Rezania *et al*, 2011).

Quality control of Extraction LPS

Quality control of the obtained LPS was done to detect the presence of any impunities in extracted LPS in special concern the protein content and nucleic acid content as follow :

1. Sodium dodecyl sulfate polyacrylamide fel electrophoresis was performed, on 12% polyacrylamide, gels as previously described by Laemmli (1970). Also the

whole cell, pellets, were suspended in 20 mL of -PB-S and boiled at 100°C for 5 min in 5 mL of 5x SAB buffer. The SDS-PAGE gels were electropholysed using 1X SDS-running buffer at constant 150V for 1hr. Protein, bands were visualized by Coomassie, brilliant, blue R-250.

2- Silver, Commassie, Blue and Ethidium, Bromide Staining : The, purified, LPS was solubilized in sample buffer to the desired concentration (1 mg/mL) and boiled for 5 min. 16ìL/well from, each sample was separated, on 12% SDS, gel, under reducing condition at 100 mA and 180 volt, Silver and coomassie, blue staining of the gels was performed according to the standard protocol.

Animals experimental design

1. Preparation of Doses

1. Bacteria : *S. typhimurium* were adjusted at a concentration of 3.8×10^9 cell/200 µl PBS using McFarland method. (Flvio *et al*, 2000).

2. Lipopolysaccharide (LPS) Solution : Partially-purified LPS solution was adjusted at aconcentration of 50 ìg/200 µl P-B-S (Sanjay *et al*, 2004).

3. Adjuvant prepararion : Thin layer of propolis was subjected to UV irradiation by a low-pressure Mercury-Vapor discharge lamb (36, Philips, VSA) with tubular.

Glass envelop that emits short - wavelength UV of radiation (254 nm) for 15 minutes at distance propolis Adjuvant investigation and according to the manufacture recommendation. Excess propolis was soaked in 95% ethanol.

The supersaturated solution was stirred for 2 hour then left to decant for another 2 hour. The supernatant preparation was kept at room temperature for uses in doses 50 µg/200 µl PBS (Omnia *et al*, 2015).

Laboratory animals and Vaccination protocol

Eighty male of albino mice (*Swiss mice*) weighting 28±4 g were, purchased from the animal, house of the Faculty of Science, Al-Kufa University. Animals were handled in accordance, with the principles of laboratory, animal care according to National Institute of Health, (NIH) guide for laboratory, animal, welfare. The mice were, housed in standard, cages where food and water were provided. Cages are maintained at a temperature, of 22 ± 2°C, relative humidity of 40-60% with a 12- h/-12 h light-/dark cycle and free access to pellet diet and water spontaneously. After two weeks of acclimatization, Mice were divided- into Four- groups a (each- group have 20 mice) and treated as follow :

Group A : Mice were interapretoneally vaccinated with mixture of LPS and Propolis.

Group B : Mice were interapretoneally vaccinated with LPS.

Group C : Mice were interapretoneally vaccinated with Propolis.

Group D : (non vaccinated control) Mice were received sterilized PBS.

Initially, primary vaccination were administrated at week 0 and followed by three booster vaccinations at week 2, week 4 week 8 and then mice were challenged with (3.8×10^9 cells/ml) of *S. typhimurium* strain.

Animal scarification

Animals were sacrificed at the end of vaccination peroid dose and the challenge test, through cervical dislocation. Blood samples from individual mice were collected at the end vaccination and challenge by aseptic heartpuncture from the heart, into commercial tubes without anticoagulant for immuinological study. It was performed by centrifuged- at 4000 rpm for 15 min, serum separated and stored at -20°C (Dacie and Lewis, 1991).

Dot blot protocol

Dot blot protocol was carried out according to a procedure reported previously by Monroe (1985). The region which will be blotted being marked by pencil on nitrocellulose membrane- BI-O-R-AD, Trans-Blot, etc). the procedure as follow :

1. It is using narrow-mouth pipette tip, spot 2ìL of Salmonella typhimurium lys at 25Mg/ml) onto the nitrocellulose membrane at thecenter of the grid has been used thearea that the solution penetrates (usually 3-4 mm diam.) has been minimzed by applying it slowly. The membrane dry has been let till it become dry.

2**.** The non-specific sites have been blocked by soaking in 5% BSA in T-BS (0.5-1 h, RT). 10cm Petri Dish for reaction chamber has been used.

3. Incubate with primary antibody (dilution for antisera, 1:640 to "1:10000" dissolved in BS-A/TB-S-T for 30min at R-T and Three times washing with TB-S-T (3 × 5 min).

4. The membranes were incubated in anti-rat secondly antibody HRP using dilution of 1:15.000 in 20 mL TBS.

5. Three times washing with TB-S-T (15min × 1, 5min × 2), then once with TB-S (5min).

6. Incubate = with ECL reagent for1min, cover-with Saran wrap (remove excessive solution from the surface), and expose X-ray film in the dark-room. Try several different- lengths of- exposure.

RESULTS AND DISCUSSION

Incidence of *Salmonella* according to specimens types

A total. of 75 specimen have been investigated in this study, depending on morphological characteristic (Fig. 1), APi 20E test (Fig. 2) and *16S rRNA* amplification (Fig. 3) results showed out of 75 studied specimens of human, 14 specimens were *Salmonella* positive (18.66%).

Table 1 : Isolation rates of *Salmonella* spp. from collected specimens.

Source of specimens	No. of examined specimens	No. of positive specimens	% of positive specimens
Human	75	14	18.66

The importance of Salmonellosis in public health sector is a growing concern day by day throughout the world and over the last several decades there have been significant shift in predominant *Salmonella* serovars associated with human infections (Steven *et al*, 2011). Enteric infection with *Salmonella* spp. is an important cause of diarrheal disease worldwide, but the frequency of the infection shows variations between studies. So, the results of this study are more or less in agree with the findings of, the previous workers, who also conducted research investigation on *Salmonella* from human stool. For example Kagirita *et al* (2017) were isolated 69 salmonella isolates, 25 (36%) from sporadic human clinical cases, 23 (34%) from human epidemic outbreaks.

Regarding of local study our results disagree with results that obtained by Mezal *et al* (2016) whom found (8%) from Children fecal samples were *Salmonella* spp positive. It is difficult to compare the prevalence of *Salmonella* among different studies, because the difference may be associated with geographical differences, sampling seasons, sample types, technical limitations of the laboratory of the study, hygienic, environmental. Accordingly, understanding the burden of pathogen specific diarrheal disease and the variation by region is important for planning effective control programs for the overall reduction of diarrhea disease among persons of all ages. Animals partly as potential source of clinical salmonellosis.

Biotechnological production of LPS

The16Sr RNA product of isolate (S1) that as processed through Finch TV software. This sequence have symbol code (H1) and sequence was analyzed using BLAST N (Basic Local Alignment Search Tool, http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi) that is an online programme belonging to NCBI (National Center for Biotechnology Information) gave results this isolate belong to *Salmonella enterica* serovar *Typhimurium* under accession number (MH109313.1). This isolates was used for such purpose as strain for the desired LPS. It was grown at standard condition, pH (7.2), temperature (37.5^0C) and time (24 hr.) on Nurientbrowth. The remarked best growth gave LPS in dry weight was 200mg/ 20 L of medium.

Quality control of the produced lipopolysaccharide (LPS)

Isolation and characterization of LPS of *Salmonella typhimurium*

Separation of LPS over SDS-PAGE gel followed by silver staining was used to detect and visually characterize the purified LPS. Sliver staining is a highly sensitive method capable of detecting as low as 1ngLPS and is routinely used for visualization of the band pattern of purified LPS. As depicted in Fig. 4A, LPS from *S. typhimurium* gave a characteristic dark staircase (ladder-like) pattern of bands. Lipid A core LPS migrating very near the dye-front stained very intensely and appeared as black region at the bottom of gel. Coomassie blue staining of the gels showed no band indicating absence of contaminating proteins (Fig. 4B). Staining of agarose gel with ethidium bromide showed no band in purified LPS products that indicate to no contamination with nucleic acid (Fig. 4C). In the present study, this procedure with some modifications was employed for extraction and purification of LPS from encountered bacterial strains; *S. typhimurium*. In our protocol, first disrupted bacterial cell wall by sonication followed by proteinase and nuclease treatment. Early treatment with the enzymes would allow early elimination of contaminating components which considerably enhances purity in the next steps. Furthermore, hot phenol-mediated extraction in the next step results in elimination of residual amounts of the enzymes added in the second step. This order of treatments would conceivably yield purified LPS with the lowest amount of contaminating protein and nucleic acids. The result of coomassie blue staining of purified LPS revealed absence of contaminating bacterial proteins suggesting the effectiveness of protein elimination by proteinase treatment and result of ethidum bromide of purified LPS revealed absence of contamination with nucleic acid.

Many novel approaches have been used for the detection of amphiphilic LPS, not all of which are functional in physiological matrices or have the required sensitivity or ease of use. One major reason for this is the failure to incorporate the amphiphilic properties of the antigen into assay design. The presentation, conformation, and host interactions of the antigens should be considered for the development of effective assays. Thus, as repositories of these necessary recognition molecules

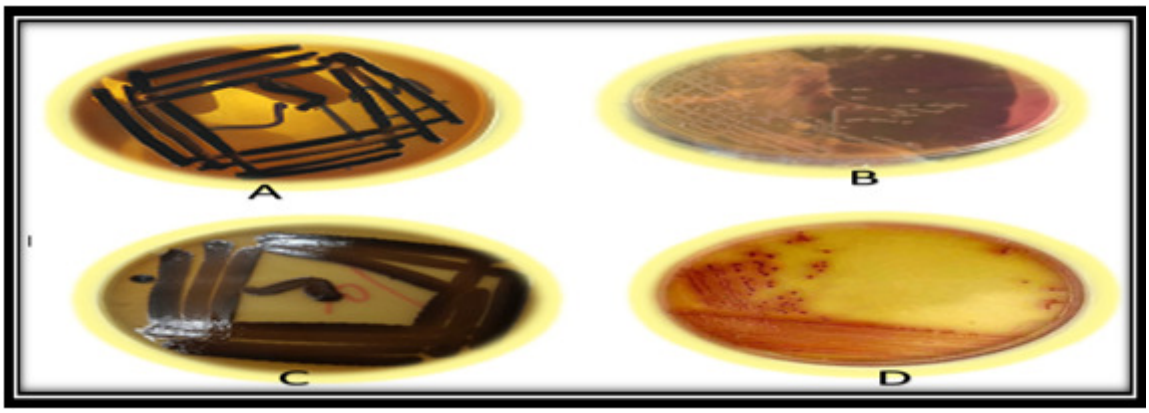

Fig. 1 : Colonies of *Salmonella* spp. isolate on: S.S agar (A) Macconkey agar (B) BS agar (C) Chromogenic agar(D).

Fig. 2 : Calculate the numerical profile in Api-20Esystem. (+): The test positive. (-): The test negative. (6704752): The numerical profile. The reading in this isolate is (-++), (+++), (—), (—+), (+++), (+-+), (-+-). Those are translated into (0,2,4), (1,2,4), (0,0,0) (0,0,4), (1,2,4), (1,0,4) (0,2,0); that equals a code of (6704752); a code that corresponds to very good *Salmonella* sp.in manufacturer's manual.

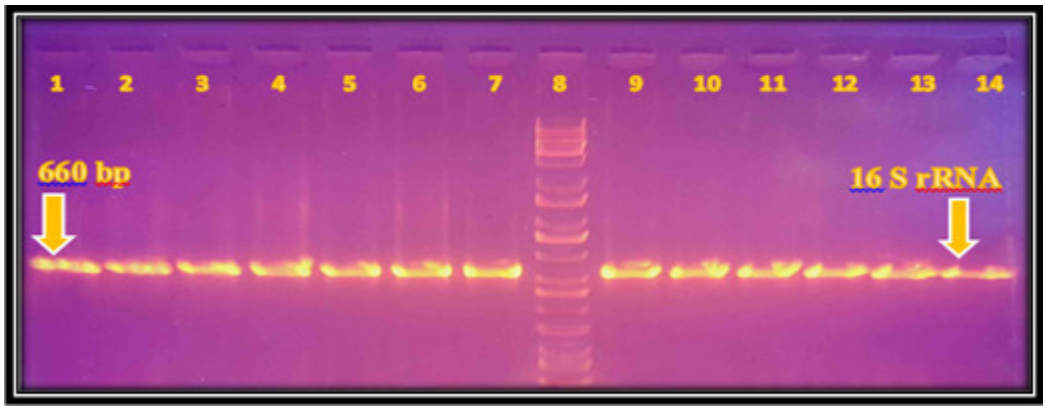

Fig. 3 : DNA amplification of a660 bp of *Salmonella* spp. detecting *16srRNA* gene using PCR. Lane 8= L adder, lane,1,2,3,4,5,6, 7,9,10,11,12,13,14 positive results.

expand to include more serogroups, so too will our ability to selectively detect (Stromberg *et al*, 2017).

Proteinase K mediated bacterial protein digestion followed by nuclease elimination of contaminating RNA and DNA and phenol-water extraction method results in highly pure LPS free of protein and nucleic acids (Apicella, 2000). So this method yielded pure LPS devoid of any protein or Nucleic acid contamination could be due to the enzyme treatment allowing early elimination of contaminating components which considerably enhanced purity in the next steps. Furthermore, hot phenol-mediated extraction subsequently might have resulted in elimination of residual enzymes (Razania *et al*, 2011). The results of silver staining clearly showed a typical ladder pattern of LPS of bands with multiple rungs obtained by Proteinase K, hot phenol-water method, which is the characteristics of smooth type of Gram negative bacteria due to the carbohydrate chain length variation of the O-antigen portion.LPS can be classified as either smooth or rough type based on the presence or absence of ladder like structure The rough form of LPS does not possess a ladder like structure due to the lack of 'O' specific chain containing repeating units of oligopolysaccharides. Variation in LPS structure forms the basis for variouschemotype observed in Gram- negative bacteria (Chart, 1995). Several attempts were made so far to introduce a dozen of techniques for

extraction and purification of LPS from strains of Gram-negative bacteria. Some techniques are technically or instrumentally demanding and so simple, cost-effective and at the same time reliable methods for isolation of pure LPS would be of great value (Perdomo and Montero, 2006). Because of the presence of contaminating substances, which are introduced to the final purified LPS during extraction and purification process and their interfering effects in most downstream immunological and biological experiments, a practical approach for their elimination is necessary. Potential contaminants include capsular polysaccharide, nucleic acids and proteins, particularly outer membrane proteins that have high potential to bind LPS with high affinity (Hitchcock and Morrison, 1984). Considering our results we showed that the, extraction of LPS by Proteinase-K-hot-phenol-water method was tedious, needed meticulous handling of phenol but yielded LPS of high purity which ensures its reliability in downstream application.

Dot blot immunoblotting and detection of immobilized antigen preparation

Dot blot was used to detected the antibody titer for the sera of intraperitoneal (IP) vaccinated animals, the primary optimization experiments confirmed that anti-serum. 1/10, 1/20, 1/40, 1/80, 1/160, 1/320 and 1/640, nitrocellulose paper coating by 25 µg/ml of *Salmonella typhimuruim* lysate.

This study was focused on identifying an effective host immune response to PLPS and it demonstrated via Dot blot technique the possibly this complex to enhance the host immune response to invading *S. typhimurium* infection and we reasoned that the lack of efficacy in previous attempts to develop a component vaccine against *S. typhimurium* may have been due to this pathogen ability to modify its surface components, including LPS and many membrane constituents once inside host cells macrophages. The results in this study showed the most sample of vaccinated animals gives result as high titer antibody and we found that serum (polyclonal antibody) of mice immunized by LPS and PLPS able to recognize each (Ag-Ab complex) by using dot blot technique (Fig. 5). Therefore, PLPS can elicit host immune responses to *S. typhimurium*.

LPS is potent activator of immune system capable of triggering cytokine release from cells of different origin. also act as a B –cell mitogen stimulating the polyclonal differentiation and multiplication of B-cells and the secretion of immunoglobulins, especially IgG and IgM (Hawkins *et al*, 2013). So, it is widely used as an inducer of TLR-4 signaling pathway and as a mediator of dendritic cell maturation (Ferreira *et al*, 2015). Therefore, it is possible to hypothesized that the improved host defense of PLPS vaccinated mice is mediated by immune mechanisms, in which macrophages and neutrophils influenced a critical role in controlling invading pathogens by phagocytosis and production of antimicrobial proteins. This has been demonstrated by Christensen *et al* (2011), (but with use another adjuvant Liposome), who showed that LPS+LIP vaccinated mice showed increased resistance to *S. typhimurium* and that this resistance was caused by increased peritoneal accumulation of neutrophils. Furthermore, it has been demonstrated an elevated influx of polymorphonuclear cells in the peritoneal cavity and this has been suggested to contribute to enhanced control of bacterial replication and thus, increased resistance. Furthermore, LP Sconjugates are potent B-cell mitogens and also activate T cells to produce IFN and TNF and thereby enhance cellular immune responses. The major structural element responsible for their toxicity and adjuvant effect is Lipid A. In low acid conditions, lipid A can be hydrolysed to obtain monophosphoryl lipid A, a compound which retains the adjuvant activity of Lipid A with reduced toxicity, and the role of propolis in this regard may be is extend the half-life of antigens in blood ensuring a higher antigen exposure to antigen presenting cells after vaccination (Dejager *et al*, 2010).

From another hand, the adjuvant action of propolis in the stimulation of phagocytosis may reasoned by the fact that propolis possess many of the characteristics associated with inducers of cell-mediated responses. The association of antigens with propolis provides a means of targeted delivery of the associated antigens directly to antigen processing cells of the reticuloendothelial system Further, the incorporation of LPS into propolis renders the soluble LPS molecule particulate and much more hydrophobic. Furthermore, it has been shown that incorporation of LPS into proplis reduced the adverse biological effects of lipid A. Therefore, it is likely that theP LPS complex is considerably less toxic than the equivalent amount of free LPS After endocytosis of the P LPS complex, it is assumed that disruption of the propolis must occur within the phagolysosomes to expose free LPS molecules for processing. This, along with the fact that nearly the entire antigenic mass is concentrated in the macrophage population of the reticuloendothelial system, may result in a longer retention time of the LPS and therefore prolonged antigenic stimulation (Hashioka *et al*, 2007).

Accordingly, it is possible to suggest that PLPS conjugate enhanced humoral immune responses; these

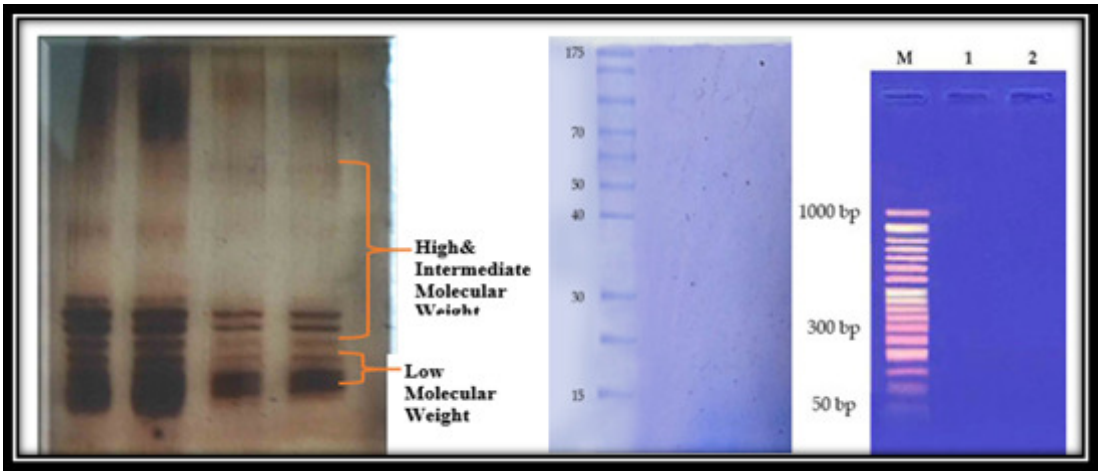

Fig. 4 : Silver, Coomassie blue and Ethidum bromide stainings of purified LPS. LPS from *S.Typhimurium*(Lanes1,2,3,4A) was purified by modified hot phenol-water extraction method and fractionated by SDS-PAGE electrophoresis followed by silver, Ladder pattern of LPS banding which is characteristic of smooth gram-negative bacteria is seen (Lanes A). Coomassie blue staining (Lanes B). The absence of band in coomassie blue staining as shown in (Lanes B) indicates no contamination of purified LPS with bacterial proteins. Residual nucleic acid contamination in purified LPS product was traced by ethidium bromide staining (Lanes C). Absence of band in LPS from *S.typhimurium* indicates no contamination with nucleic acids in purified LPS products.

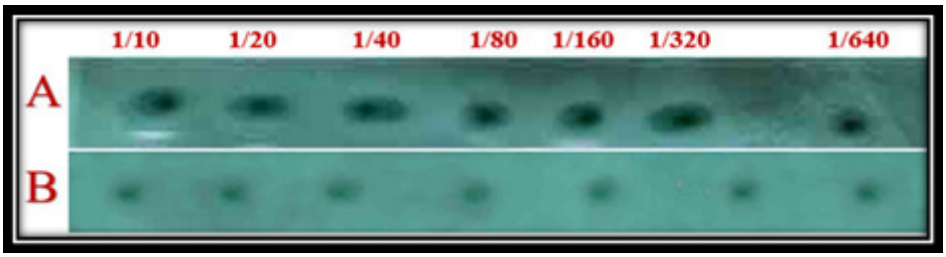

Fig. 5 : Dot blot of *S. typhimurium* lysate with serial dilution polyclonal antibody of (A) PLPS and (B) LPS**.**

findings can be justified by increased internalization of hydrophobic lipid antigens by macrophages that ultimately improved antigen presentation to cells. These macrophages function as antigen-presenting cells, which take up antigen, catabolize them and express the antigenic determinants in an energy requiring process before being presented to antigen specific T-cells (Chhibber *et al*, 2004). It is also possible that these PLPS activated macrophages contribute towards positive regulating effects on the induction of specific immune response. Thus, the results of the present study elucidate that incorporation of *S. typhimurium* LPS into Propolis not only makes it possible to achieve a considerable immune response to lipid A-based immunogens by circumventing toxicity associated with lipid A, but also to convert polysaccharide antigens into thymus-dependent antigens.

Finally, *Salmonella* infections are a major global health burden, as they cause high morbidity and mortality worldwide. Strategies that prevent *Salmonella*-related diseases are greatly needed, and there is a significant push for the development of vaccines against non-typhoidal *Salmonella enterica* serovars. The lack of an NTS vaccine is exacerbated by the widespread presence of multiresistant clinical isolates, making the treatment of NTS infections even more difficult (Crump *et al*, 2015).

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